LONG-TERM ORGAN CULTURE OF THE SALAMANDER HEART

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ABSTRACT

Beating salamander hearts were maintained in tissue culture for periods ranging from 1 to 6 months. After 1, 3, or 6 months of culture, six hearts, along with six control hearts, were fixed for electron microscopy.

In control tissue, the sarcoplasmic reticulum usually demonstrated the normal pattern of paired, linearly arranged membranes, although in some cases, the reticulum showed a variation from these membranes to a series of small vesicles. There was no evidence of a T-system of tubules in any of the material examined. Desmosome–Z band complexes were observed in almost all sections of both control and experimental material. A possible role of these complexes in the excitation-contraction mechanism is discussed.

In 3 month cultured material, alterations in normal myofibrillar pattern occurred. Small segments of myofibrils branched from one Z band to join the Z band of an adjacent myofibril, or appeared to be fraying out into the sarcoplasm. In 6 month cultured material, myofibrils were fragmented into short segments from which myofilaments frayed out into the sarcoplasm. This filamentous material may be remnants of myofilaments.

Despite the morphological changes in myofibrils, the heart pulsation rate, established at the beginning, was maintained throughout the culture period. It is suggested that the alterations, observed in the experimental material, occurred in elements not essential for heart beat maintenance, or that these alterations have not yet progressed to a critical point of affecting the heart beat.

INTRODUCTION

Ever since the development of tissue culture techniques, most muscle types have been the subject of much investigation. In recent years dissociated embryonic and adult heart cells and small pieces of embryonic and adult heart tissues have been cultured and studied (2, 3, 5, 10, 20, 31).

During a study of the growth of various urodele tissues in vitro in our laboratory, whole hearts from mature larval salamanders were maintained in culture for varying periods of time. In culture, the hearts established pulsation rates different than those they had had in the intact animal. Some hearts had a slow rate of beat (2–15 beats per min), while others had a more rapid rate (45–60 beats per min). There were small temporal fluctuations in the daily rate of beat which suggested a 24 hr endogenous rhythmicity in the pulsation rates. The pattern of beat established at the initial time of culturing continued throughout the culture period. Pulsation patterns varied in the cultured hearts as follows: (*a*) some whole hearts pulsated as a single unit; (*b*) in others, only one portion pulsated, usually the ventricle; and (*c*) in still others, a contraction in the auricle

was followed by a contraction in the ventricle. The rhythmicity data will be reported in a future communication.

The present report will deal with the progressive morphological changes observed on an ultrastructural level in whole hearts that were maintained in culture for periods of up to 6 months.

MATERIALS AND METHODS

From a colony of larval salamanders (*Taricha torosa*), all of the same age, 50 animals were used in this project. Whole hearts from half of these animals were removed and placed in tissue culture, while the remainder were used as controls.

Beating whole hearts of the experimental group were aseptically removed from anesthetized animals. Then, they were placed immediately into a balanced salt–receiving solution containing, per milliliter of solution, 1000 units of penicillin G, 1000 μg of streptomycin, and 100 units of Mycostatin. The beating hearts were transferred into Eagle's medium (4) containing 10% fetal bovine serum. 1 drop of chicken plasma was placed on a cover slip, a heart was placed on this drop, and then a drop of chick embryo extract, diluted to 20%, was placed onto the explant. After the plasma clot became firm, the culture was incubated overnight at 25°C. The following day, 0.75 ml of Eagle's medium was added to the culture tube without disturbing the plasma clot. Incubation was continued at 25°C in a 5% CO_2 atmosphere for varying lengths of time. The medium was changed routinely three times a week. The explants were checked daily and the beat rates were recorded two or three times during the week at various times of the 24 hr day.

At the end of 1, 3, and 6 months, six hearts from the cultured group and six hearts from the control group were fixed for electron microscopy. Both the control and the cultured hearts continued to beat two or three times after being placed in a fixative, thus aiding in internal fixation; but the hearts were fixed in a contracted state. The fixatives employed were either (*a*) 2% OsO_4 buffered with 0.06 M KH_2PO_4-K_2HPO_4 (1), or (*b*) 4–6% redistilled glutaraldehyde buffered with cacodylate (26), all being buffered to a pH of 7.4–7.6. Tissues that were placed into solution (*a*) were fixed for 1–1.5 hr at approximately 4°C. Tissue placed into solution (*b*) were fixed for 12–18 hr at approximately 4°C, washed in cold 7% sucrose-cacodylate–buffered solution, and stored at 10°C in this sucrose mixture for at least 24 hr. Then, the tissues were washed in fresh buffer and postfixed in 2% cacodylate-buffered OsO_4 for 1–1.5 hr. at 4°C. All tissues were dehydrated and embedded according to the Epon 812 technique (18), using an Epon mixture (Shell Chemical Co., New York) of 6 parts of mixture A:4 parts of mixture B, plus 0.20 ml of accelerator (DMP-30) per 10 ml of embedding medium. The capsules were hardened from 36–48 hr in an oven at 60°C.

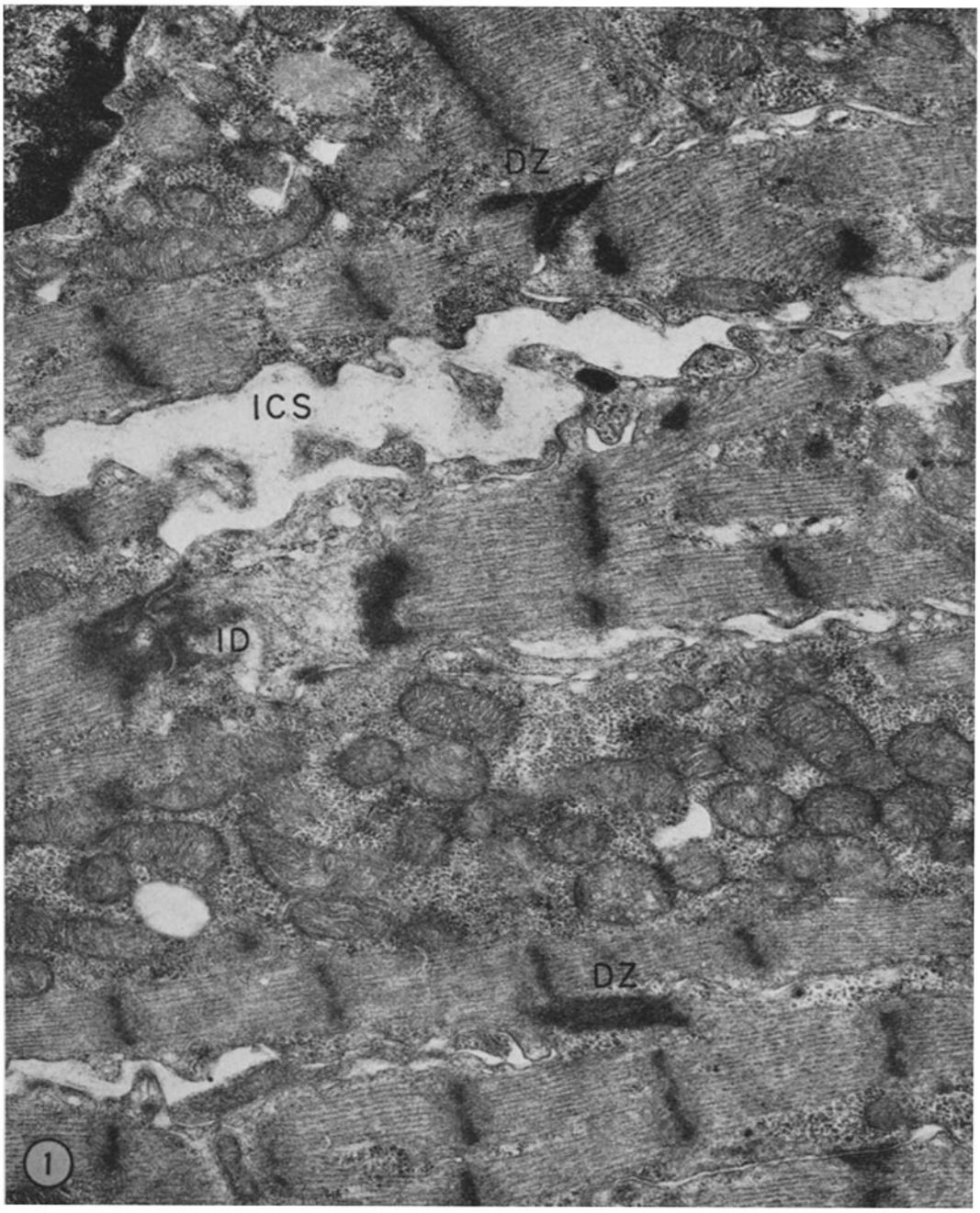
The embedded tissues were sectioned with glass knives on a Porter-Blum MT-1 ultramicrotome and sections were collected on uncoated 200-mesh grids. Some tissue sections were stained in a saturated aqueous solution of uranyl acetate for 2–3 hr, and others were stained in the uranyl acetate solution for 1 hr and then in a lead citrate solution for 10–15 min (21). The sections were viewed in an RCA EMU-3H at initial magnifications ranging from 4000 to 40,000.

OBSERVATIONS

In tissue sections from the normal control salamander hearts, the general morphological pattern observed in cardiac muscle from the turtle (9), toad (11), and frog (24) was confirmed. In the salamander, the muscle cells were usually in close proximity to one another, but at intervals the cardiac cells of both the ventricle and, particularly, the auricle were separated, giving rise to intercellular spaces. These spaces communicated with one another and with the subendothelial space. The latter space usually contained collagen fibers, in addition to, at various levels, fibrocytes, unmyelinated nerves, and white blood cells. Sections from the auricles and the ventricles of at least 14 control animals exhibited the usual striations plus branching and anastomosing of cardiac myofibrils and intercalated discs (Fig. 1). In the same figure, desmosomes (maculae adherentes) (6) had a highly electron-opaque matrix situated in the sarcoplasm of apposing cells. The matrix extended for varying distances along the sarcolemma and connected at regular intervals with Z bands in adjacent myofibrils.

Periodically throughout the sarcoplasm of the control ventricles the smooth reticulum was transformed from the usual system of linearly arranged membranes into a series of vesicles (Fig. 2). The vesicles formed concentric circles around organelles or were seen throughout the sarcoplasm as a linearly arranged single or multiple series. Occasionally, a single or double series of linearly arranged vesicles were observed in the auricular sarcoplasm. The authors are studying these vesicular structures further in an attempt to understand their formation and their function.

In the ventricular tissue of the control hearts, discrete vesicles were observed in the sarcoplasm. These vesicles (Figs. 3 *a* and 3 *b*) varied in form and size and were limited by a single membrane.



FIGURES 1–3 *a*, and 3 *b* Sections from control salamander cardiac muscle. The material shown in Figs. 1 and 2 was fixed in 4–6% redistilled glutaraldehyde in cacodylate buffer and postosmicated; that of Figs 3 *a* and 3 *b* was fixed in osmium tetroxide in the potassium phosphate buffer.

FIGURE 1 This section, from a ventricle, illustrates an intercellular space (*ICS*), an intercalated disc (*ID*), and two desmosome–Z band complexes (*DZ*). The sarcoplasm between the myofibrils contains many mitochondria. ×12,000.

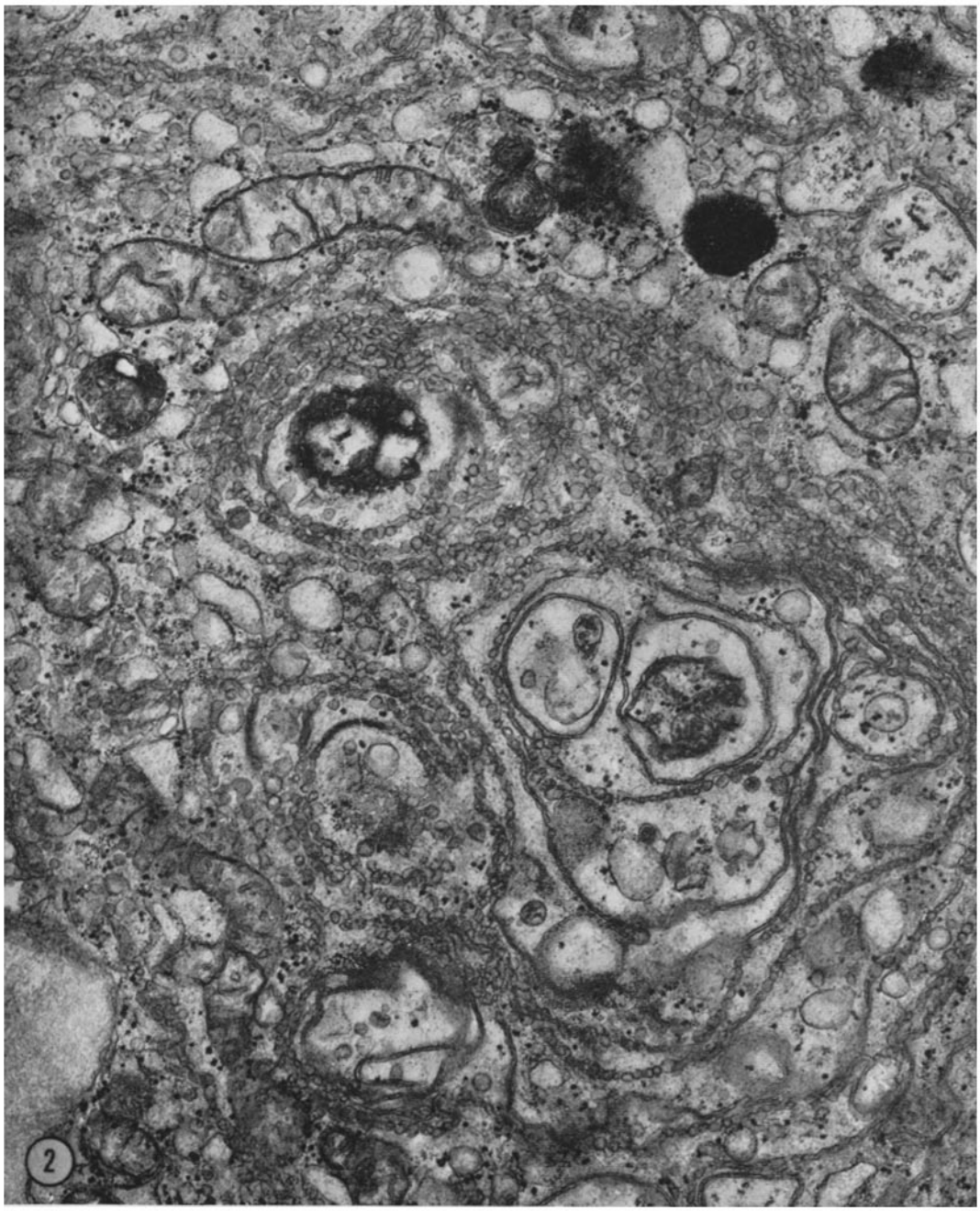
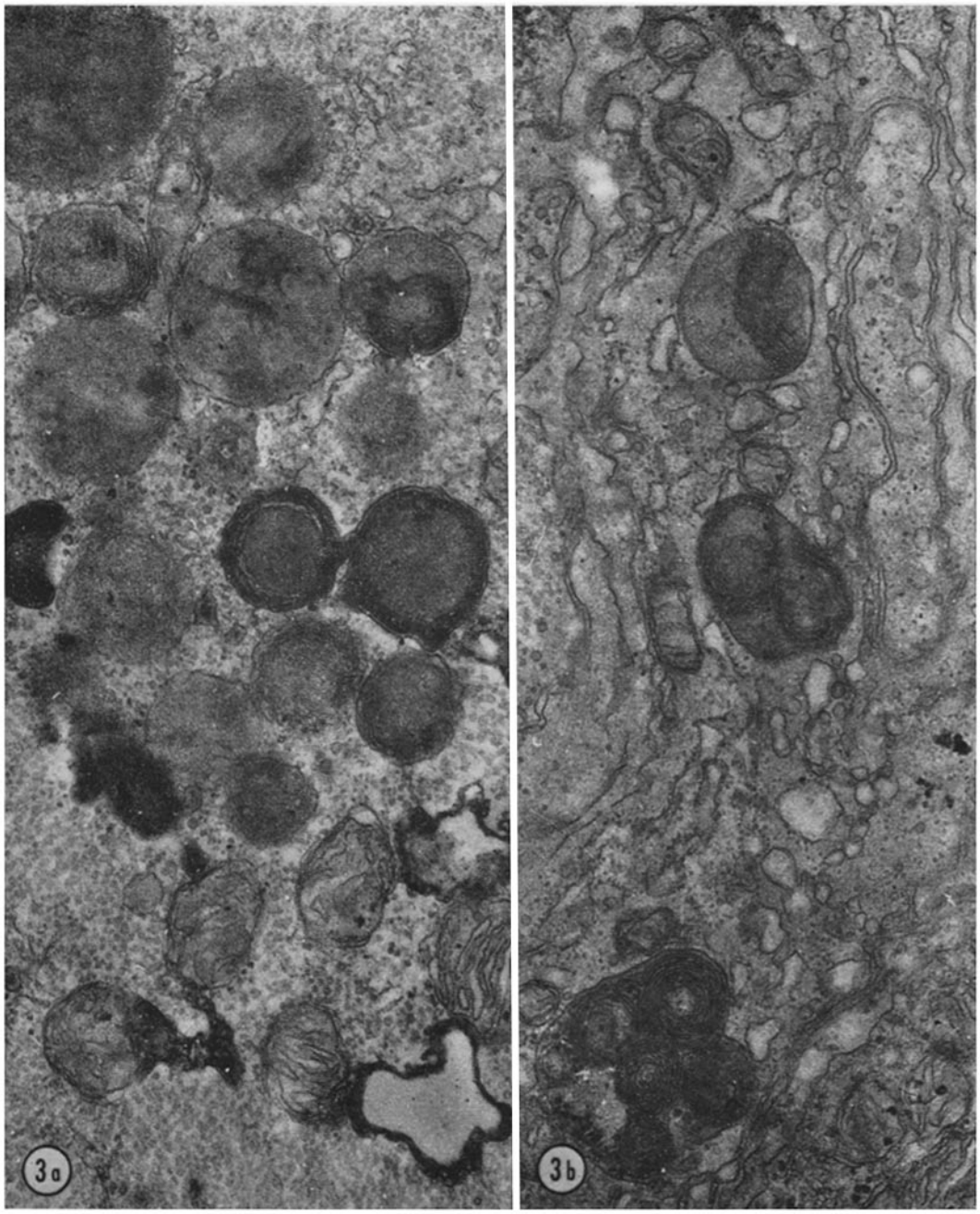


FIGURE 2 The smooth sarcoplasmic reticulum, as represented in this section from a ventricle, was modified from its usual linear arrangement to a series of small vesicles which were usually observed in concentric circles around organelles. ×18,000.



FIGURES 3 *a* and 3 *b* These two photographs, of sections from two different ventricles, illustrate the discrete vesicles with their single limiting membrane and the various structural configurations within these membranes. ×20,000.

This limiting membrane contained: (*a*) a uniformly dense material; or (*b*) many concentrically arranged membranes which did or did not have a centrally located light core; or (*c*) short rodlike structures; or (*d*) a combination of (*b*) and (*c*). These vesicles may be lysosomes in various stages of maturation. An initial survey of the acid phosphatase activity in the control muscle cells indicated the lysosomal nature of these bodies. In our laboratory many enzymatic reactions have been demonstrated in these vesicles and in the cardiac cells, and will be the subject of a future report.

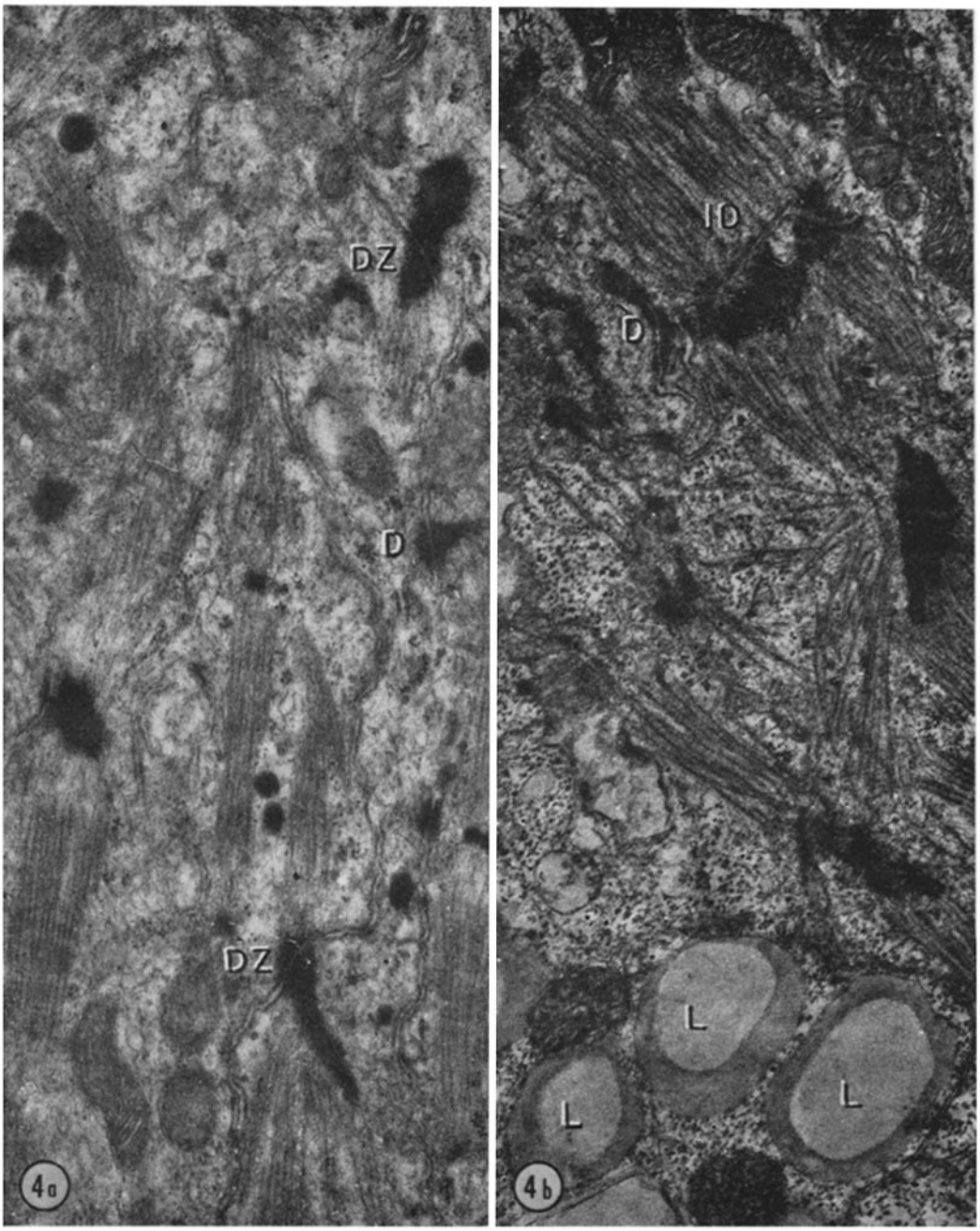
Electron micrographs of sections from three different hearts, cultured for 3 months, are illustrated in Figs. 4–6. The first indication of any morphological alteration in the myofibrils appeared at this time. The sections of the 1 and 2 month cultured tissue did not demonstrate any differences in the myofibrillar morphology from sections of the control tissue. However, in the 3 month cultured material, particularly in the auricles, the myofibrils were broken up into short segments from which single or double units of myofilaments branched off. These units appeared to anastomose with adjacent myofibrils or to extend into the surrounding sarcoplasm (Fig. 4 *a*). In some sections the branching myofilaments extended from the Z band of one myofibril to the Z band of an adjacent myofibril (Fig. 4 *b*). In addition to the usual intercalated discs, desmosomes, lipid granules, and desmosome–Z band complexes, there were electron-opaque granules (Fig. 4 *a*) which were surrounded by a single limiting membrane.

In the ventricles, a variety of myofibrillar patterns was seen. First, there were extensive areas where the myofibrils were intact, but in these areas there was considerably more branching and anastomosing between adjacent myofibrils (Fig. 5 *a*) than was usually observed in normal cardiac cells. Secondly, in many sections myofibrils were divided into smaller units, and their myofilaments fanned out from a Z band to join either an adjacent myofibril or other Z bands (Fig. 5 *b*). Thirdly, there were areas that exhibited little or no intact myofibrils (Fig. 6). From short segments of myofibrils, the myofilaments appeared to be fraying out into the sarcoplasm. Intercalated discs, desmosomes, and desmosome–Z band complexes were observed along the sarcolemma of the muscle cells in the ventricles. The electron-opaque material of the intercalated discs (Fig. 6) extended for varying distances into the sarcoplasm.

Material from the hearts cultured for 6 months is illustrated in the remaining four electron micrographs. These sections are from three different ventricles. Typical areas with a few intact myofibrils and some short myofibrillar segments which sometimes contained Z bands are demonstrated in Fig. 7 *a*–7 *c*. The sarcoplasm was filled with filamentous material. Many pinocytotic vesicles, either in the sarcoplasm adjacent to the sarcolemma or in the process of being budded off from the sarcolemma into the sarcoplasm, were observed. The intercellular spaces were filled with collagen fibers (Fig. 8) which were much more evident than in the sections from earlier cultured materials. In Fig. 7 *c*, showing a section from the same specimen seen in Fig. 7 *a*, a portion of an intact myofibril is demonstrated. From certain points (arrows) along this myofibril, myofilaments are seen fraying out into the sarcoplasm. In Figs. 7 *a* and 7 *b*, myofilaments (arrows) can be seen fraying out into the sarcoplasm from segments of myofibrils. Filaments of the same caliber as those fraying out from the myofibrils can be seen throughout the sarcoplasm. In the sarcoplasm of the 3 and 6 month cultured material the sarcoplasmic reticulum was very sparse, and in some sections of the 6 month cultured material there appeared to be none at all.

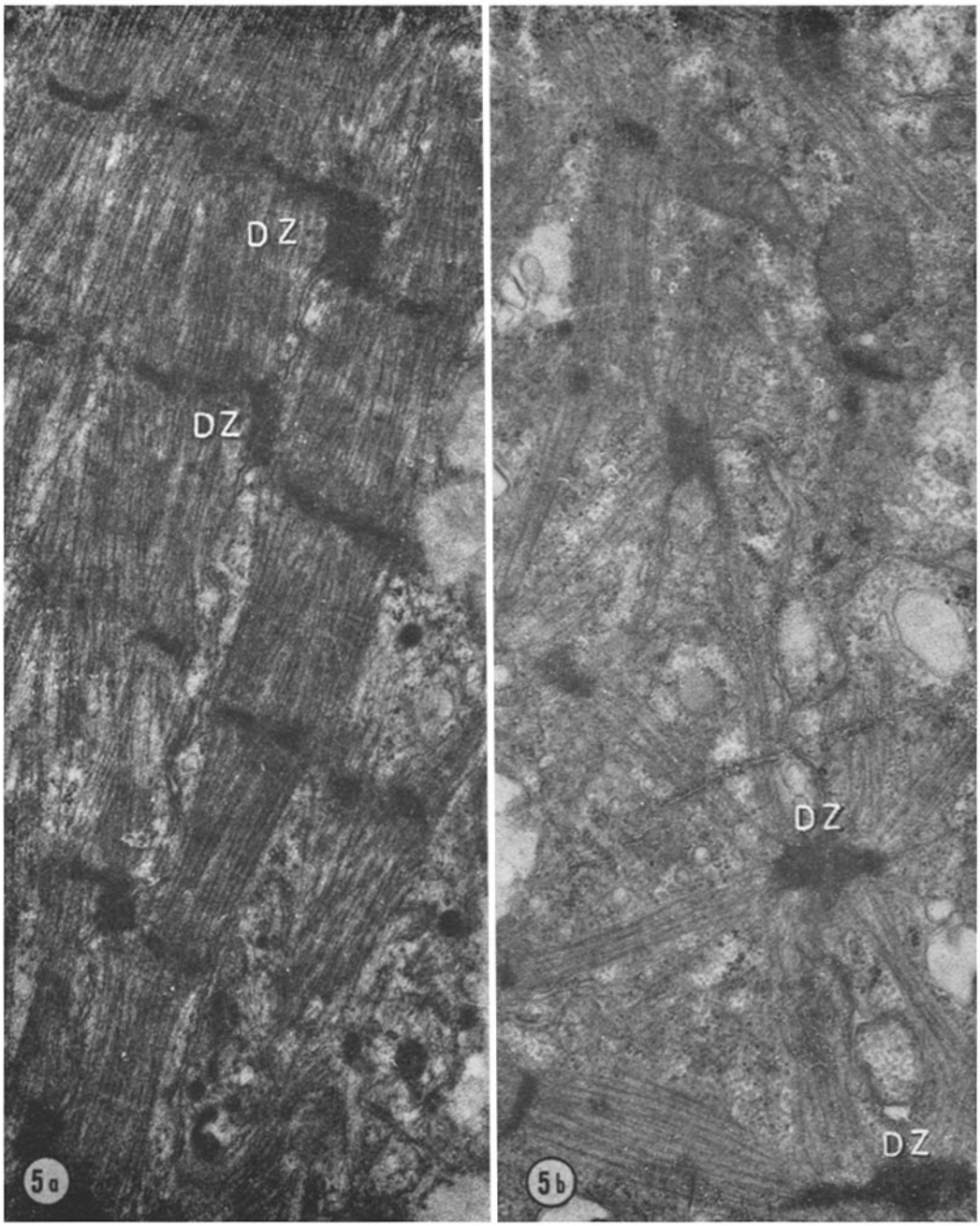
DISCUSSION

The sarcoplasmic reticulum was observed regularly in the ventricles of the control cardiac tissue, and only occasionally in the auricles of the same tissue. In contrast, the sarcoplasmic reticulum was greatly reduced in the cultured material, particularly from the 3rd and 6th month series. In many sections it was not visible in any form. In review articles by Smith (25), Fawcett (7), Fawcett and McNutt (8), Huxley (14), Sonnenblick and Stam (27), and Stenger and Spiro (30), in which the reticulum of muscle cells is described and discussed, specific mention is made of the fact that cardiac cells are a slow-acting type of muscle cell; therefore, cardiac cells require a less extensive reticulum than fast-acting cells such as those of skeletal muscle. In both the control and the experimental tissues used in this study, there was no evidence of the T-system tubules described in the forementioned review articles. The paucity of the sarcoplasmic reticulum and the absence of



FIGURES 4 *a* and 4 *b*–6 Sections from 3-month cultured whole hearts that were fixed in 4–6% redistilled glutaraldehyde, buffered in cacodylate and postosmicated.

FIGURES 4 *a* and 4 *b* These areas, from two different auricles, illustrate the segments of myofibrils radiating out in many directions from Z bands. Also shown are a desmosome (*D*), desomsome–Z band complexes (*DZ*), an intercalated disc (*ID*), and lipid granules (*L*). ×24,000.



FIGURES 5 *a* and 5 *b* These sections, from two different ventricles, demonstrate several desmosome–Z band complexes (*DZ*), the increased branching of the myofibrils, and segments of myofibrils radiating out in many directions from Z bands. ×24,000.

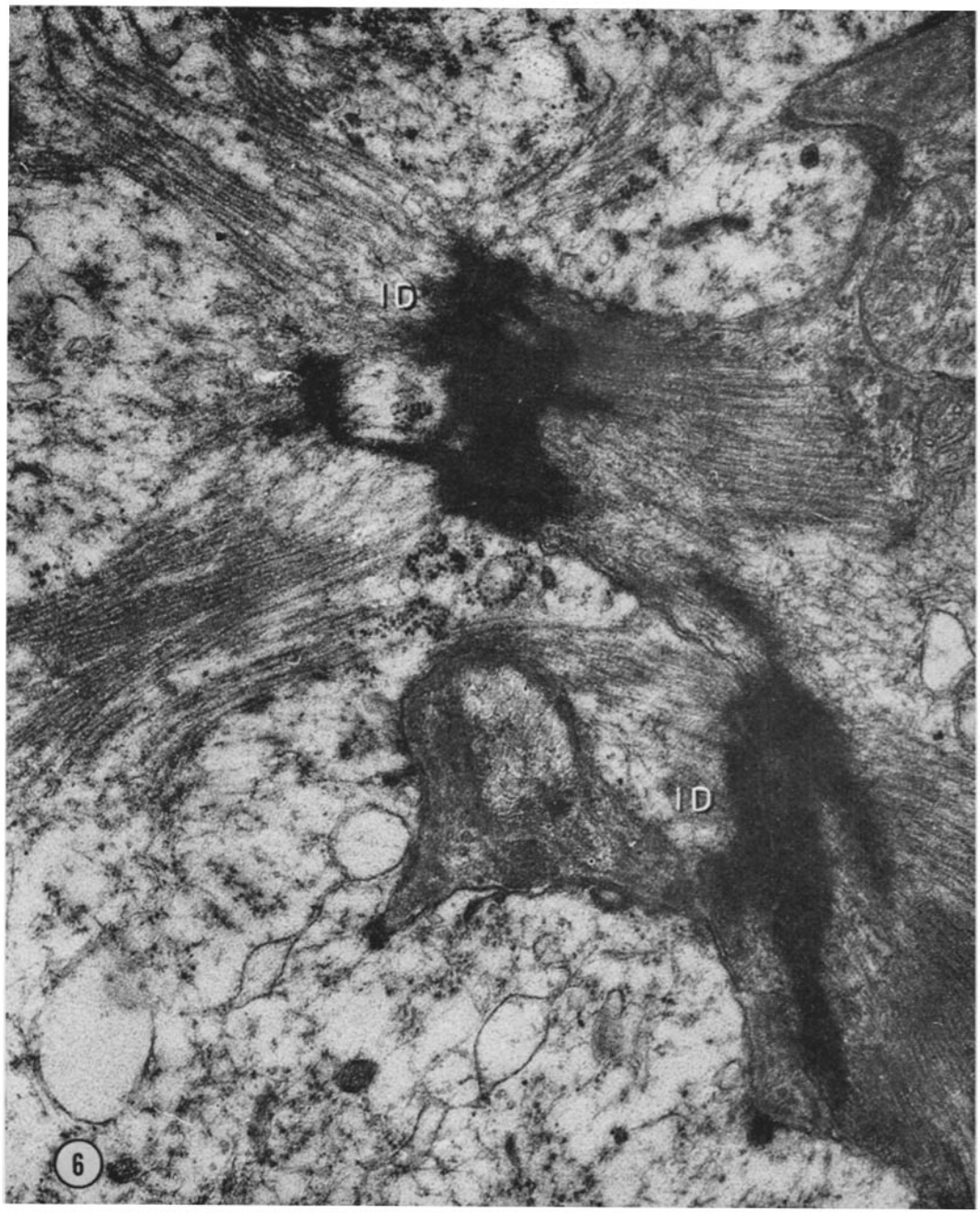
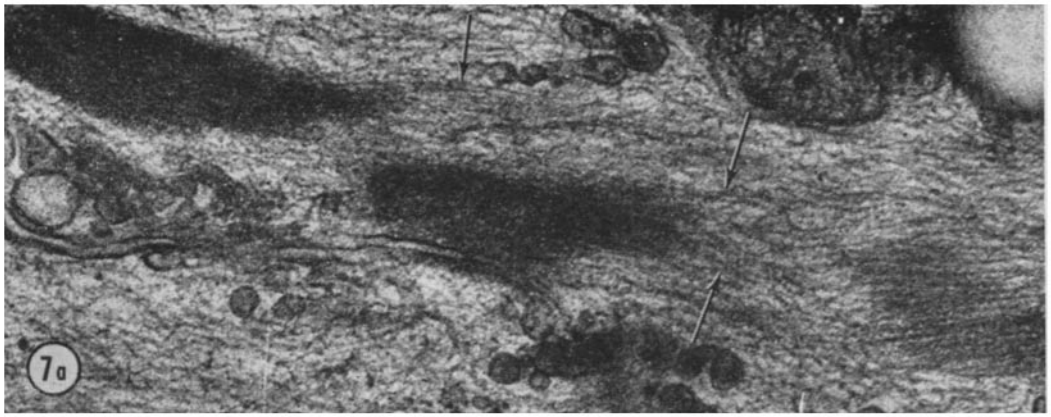
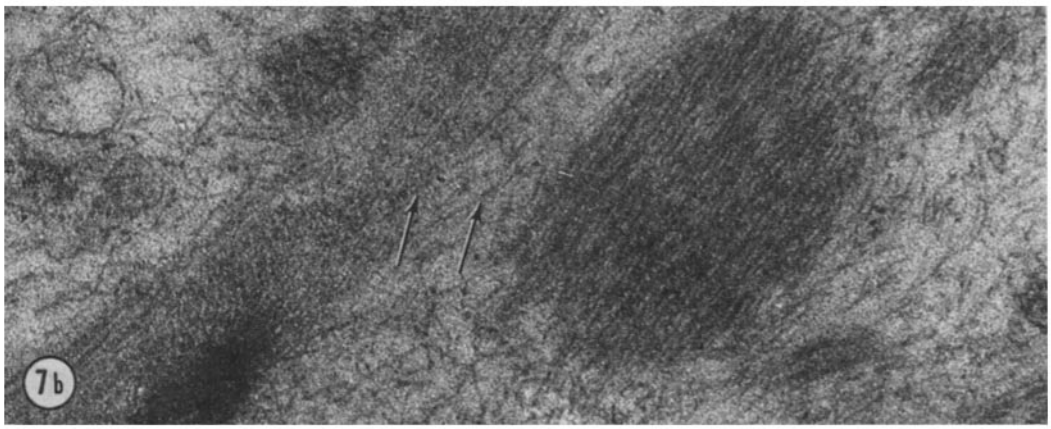
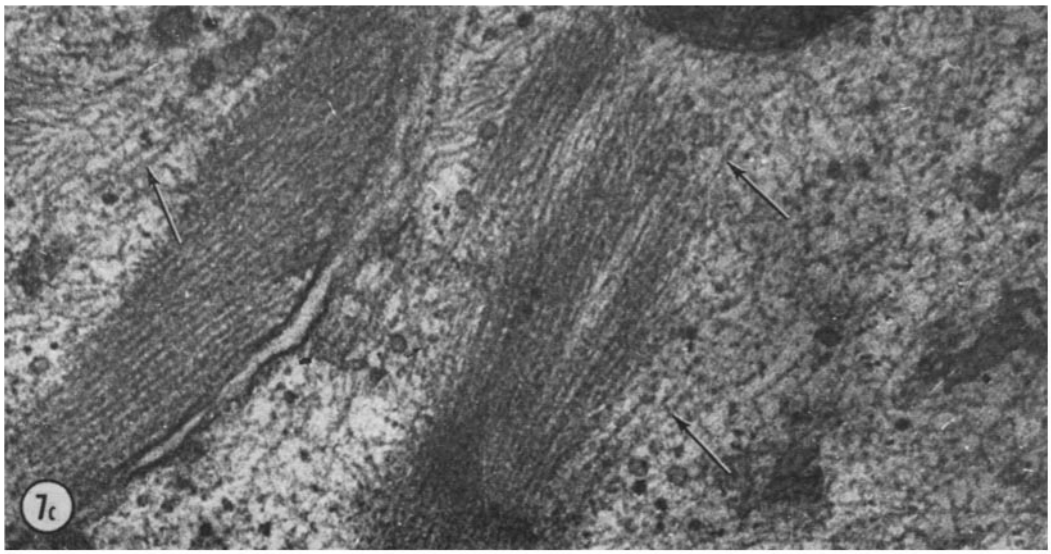


FIGURE 6 This section, from a third ventricle, shows two intercalated discs (*ID*) with electron-opaque material extending into the sarcoplasm, short segments of myofibrils, and myofilaments fraying out from the myofibrils into the sarcoplasm, along with single strands of myofilaments in the sarcoplasm. ×28,500.







FIGURES 7 *a*–7 *c* and 8 These are examples of sections from whole hearts that had been cultured for 6 months and were fixed in 2% osmium tetroxide in a potassium phosphate buffer.

FIGURES 7 *a* and 7 *b*, and 8 These sections from three different ventricles demonstrate the decrease in intact myofibrils, the increase in the filamentous material in the sarcoplasm, the many pinocytotic vesicles along the sarcolemma, and the increase in collagen fibers (Fig. 8, *C*) in the intercellular spaces. At the areas indicated by arrows the myofilaments appear to be fraying out into the sarcoplasm. Figs. 7 *a* and 7 *b*, ×35,000; Fig. 8, ×25,000.

FIGURE 7 *c* This section represents another area from the specimen seen in Fig. 7 *a*, demonstrating a portion of an intact myofibril with some myofilaments fraying out into the sarcoplasm (arrows) and the sarcoplasm filled with filaments of the same caliber as the fraying-out myofilaments. ×40,000.

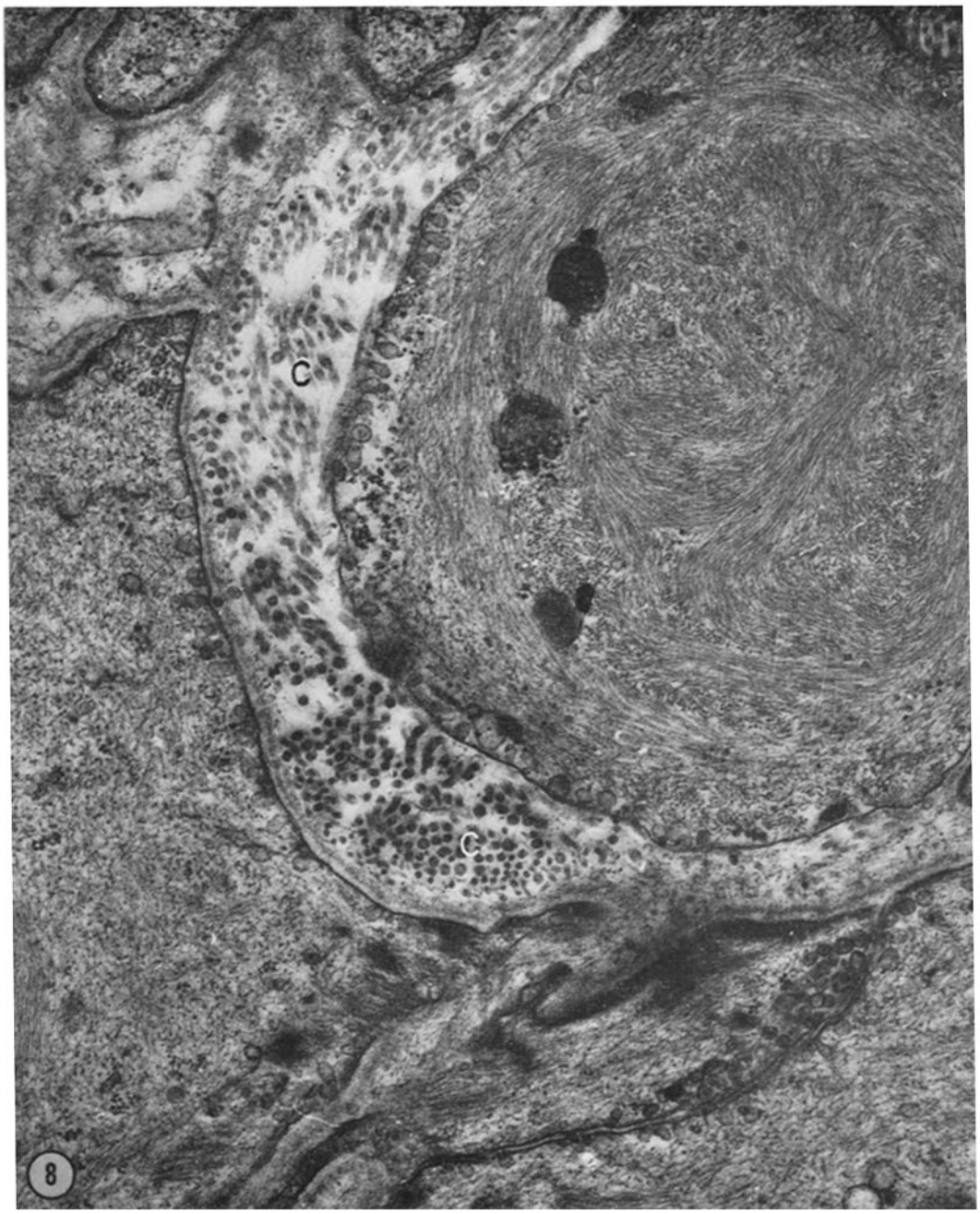


FIGURE 8

the T-system in frog ventricular muscle were reported by Staley and Benson (29). Since the majority of the cardiac tissue examined in the present study was fixed in a contracted state, the possibility of observing a triad or any extensive reticulum may have been impaired. Recently, a method of obtaining fixed control tissue in a non-contracted state has been implemented. If this

method proves successful, additional information concerning the sarcoplasmic reticulum in the control and the experimental cardiac tissues may be obtained by studying serial sections.

In our material the sarcolemma involved in the intercalated discs usually followed a straight course through the disc, similar to what has been described in the embryonic and newborn hearts of rabbits (20) and of mice (5), in the toad (11), and in the frog (22, 23, 24). In this study only larval hearts were examined, but an examination of adult heart tissue is being planned in order to determine whether or not there are any changes in the course of the cell membranes through the discs such as those that have been described in adult rabbits (20). Tight junctions associated with the intercalated discs of mammalian heart muscle have been reported; however, no such junctions were observed in the salamander heart muscle, a finding which is in agreement with the report of Staley and Benson (29).

The electron-opaque material in the sarcoplasm adjacent to the cell membranes of the discs extended for varying distances away from the discs into the sarcoplasm. It is well established that the sarcolemma and the sarcoplasmic reticulum are involved in the excitation-contraction mechanism in striated muscle (12, 13, 15, 16, 28). Since the reticulum is poorly developed in the larval hearts, the extension of the electron-opaque material of the intercalated discs might possibly provide an alternative method of conveying an impulse to the myofibrils. The desmosome–Z band complexes are very numerous in both the control and the experimental cardiac cells, and could also play a role in conveying an impulse from the sarcolemma to the myofibrils. However, these are clearly speculative ideas, and the authors are continuing their studies on the structure of the desmosome–Z band complex. The electron-opaque matrices of the Z band and the desmosomes appear to be structurally similar, as has been suggested by Fawcett and Selby (9), Muir (19), and Fawcett and McNutt (8).

The progressive morphological changes occurring after 3 and 6 months of culture have been described. Cedergren and Harary, in their study of beating single rat heart cells (2, 3), illustrated myofilaments radiating from Z bands similar to the myofilaments that we have observed in the sections of the 3-month cultured hearts. Firket, in his study of myofibrillar formation (10), described, first, single myofilaments developing in the sarcoplasm, then, short segments of myofibrils developing from these myofilaments, and finally, these short segments uniting to form the myofibrils. The illustrations of the developmental stages in the myofibrillar formation are identical with the micrographs of the degenerative patterns in the cardiac myofilaments of the 3- and 6-month cultured hearts. Lentz, in his study of striated muscle in limb regeneration (17), described stages of degeneration of adult muscle cells to a myoblast-like cell and then the regeneration of this cell to an adult muscle cell. During the degeneration of the muscle cells the intact myofibrils broke up into short segments from which the myofilaments frayed out into the sarcoplasm. In the present study, the myofibrils of the cardiac cells follow the same pattern of degeneration that is described in the limb muscle cells.

As previously mentioned, when the whole hearts were placed in tissue culture they established a new rhythm of beat which was maintained throughout the culture period. At this time, any direct correlation between the morphological changes observed in the myofibrils and the patterns of beat cannot be stated. It appears that the alterations which have occurred have been in elements not essential for the maintenance of the heart beat, or that these alterations have not progressed to a critical point at which the heart beat would be affected. Until the experimental tissue can be fixed in a noncontracted state, no speculation as to how, or if, the single myofilaments in the sarcoplasm or the short segments of myofibrils are able to contract.

The possibility exists that the repeated changing of the medium and the accumulation of metabolic by-products may have an adverse effect on material maintained in tissue culture for extended periods of time. Therefore, a different method of culturing the whole hearts has been used. The hearts are implanted in a tunnel made in the dorsal fin of a host animal. The highly vascular dorsal fin acts as a "culture chamber" and provides optimal physiological conditions for the maintenance of the cardiac tissue. To date, the beating hearts so implanted have been maintained for 3 months. It has been noted that after 1 month a sac is formed around the implant, and that one or both auricles may be attached to this newly formed sac. Sections from fixed hearts that have been implanted for 12 and 15 wk have

demonstrated that the same morphological alterations occurred in the myofibrils as described in the above experimental tissue. The implant procedure will maintain the hearts in a physiological environment which is more normal than that found in tissue culture; and, with the auricles becoming attached to the newly formed sac, a greater tension will be placed on the beating, implanted heart, thus simulating a more normal mechanical environment. Employment of this implant procedure will permit hearts to be maintained for much longer periods so that studies can be continued on the myofibrillar alterations. The effect of these alterations on the pulsation rate and on the method of contraction in the implanted cardiac muscle cells will be investigated.

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